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Mice That Express Enzymatically Inactive Cathepsin L Exhibit Abnormal Spermatogenesis[1]

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ABSTRACT

The finding of large, stage-specific changes in secretion of procathepsin L by rat Sertoli cells has led to the hypothesis that this proenzyme promotes the survival, replication, or differentiation of spermatogenic cells. Experiments described herein used a mouse model to test this hypothesis. To prove that mice are appropriate for this purpose, we first demonstrate that mature mouse Sertoli cells express cathepsin L mRNA in the same stage-specific manner as rat Sertoli cells and they also secrete procathepsin L. To test whether catalytically active cathepsin L is required for normal spermatogenesis, we examined the testes of 110- to 120-day-old *furless* mice, which express catalytically inactive cathepsin L. Morphologic examination of testes of *furless* mice revealed both normal and atrophic seminiferous tubules. Enumeration of atrophic tubules in *furless* and control mice demonstrates that lack of functional cathepsin L results in a 12-fold increase in seminiferous tubule atrophy. To determine whether lack of functional cathepsin L affects the production of male germ cells in apparently normal, nonatrophic tubules, we compared numbers in control and *furless* mice of preleptotene spermatocytes, pachytene spermatocytes, and round spermatids per Sertoli cell. Results demonstrate that the lack of functional cathepsin L causes a 16% reduction in formation of preleptotene spermatocytes and a 25% reduction in differentiation of these cells into pachytene spermatocyte. These results suggest that procathepsin L either directly or indirectly has two distinct functions in the testis. This proenzyme prevents atrophy of seminiferous tubules and promotes the formation of preleptotene spermatocytes and the differentiation of these meiotic cells into pachytene spermatocytes.

gamete biology, Sertoli cells, spermatid, spermatogenesis, testis

INTRODUCTION

There is considerable evidence that germ cells regulate gene transcription by mature rat Sertoli cells. In these somatic cells, the steady state levels of transcripts encoding a variety of growth factors, receptors, and proteases change as their adjacent germ cells progress in synchrony through the stages of the cycle of the seminiferous epithelium [1–3]. The stage-specific patterns of expression of these transcripts are diverse, suggesting that interactions of germ cells with Sertoli cells are complex and possibly important to germ cell development.

We have studied germ cell-Sertoli cell interactions from the vantage point of the stage-specific synthesis by rat Sertoli cells of the proenzyme form of cathepsin L. Transcription of the cathepsin L gene and synthesis and secretion of its proenzyme by Sertoli cells change more than 10-fold as the adjacent germ cells progress through the stages of the cycle of the seminiferous epithelium [4, 5]. As a result, whereas procathepsin L is an abundant secretory product of stage VI–VII tubules, its synthesis and secretion are undetectable at most other stages [5]. The large stage-specific changes in secretion of procathepsin L by rat Sertoli cells has led to the proposal that secretion of this proenzyme is required to maintain the normal structure and function of the seminiferous epithelium and, thus, spermatogenesis [6, 7]. That proposal has been reinforced by the observation that in vivo, this proenzyme appears to accumulate around specific types of germ cells, in step 17 and 18 spermatids [8]. In addition, studies of the expression and function of cathepsin L in cultured Sertoli cells have led to the hypothesis that this enzyme regulates the tightness of the blood-testis barrier [9]. To date, however, no in vivo experiments have examined whether a lack of functional cathepsin L in the testis has a deleterious effect on spermatogenesis.

The studies described in this paper test the hypothesis that the stage-specific secretory product of Sertoli cells, procathepsin L, promotes the survival, replication, or differentiation of spermatogenic cells. Comparing spermatogenesis in wild-type mice and *furless* mice tested this hypothesis. A point mutation in the cathepsin L gene of *furless* mice converts the glycine at amino acid 149 to an arginine, rendering the protein enzymatically inactive [10]. Although these animals are fertile, there is evidence that in rodents, sperm production can vary as much as 5-fold without affecting fertility [11]. Thus, fertility of *furless* mice is not evidence of quantitatively normal spermatogenesis. Prior to evaluating the effect of the *furless* mutation on spermatogenesis, we recognized that it was necessary to establish that procathepsin L was a major, stage-specific secretory product of mouse Sertoli cells. This fact had not been established and could not be assumed because mouse Sertoli cells secrete only minimal amounts of another major product of rat Sertoli cells, androgen binding protein [12]. Thus, the first experiments described herein establish that fact.

Having validated the use of mice to test whether enzymatically active cathepsin L is required for spermatogenesis, we compared the morphology of seminiferous tubules of *furless* and control mice. Results from this comparison

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demonstrate an increased atrophy of seminiferous tubules of *furless* mice. Furthermore, comparison of numbers of germ cells per Sertoli cell in control mice with numbers in seemingly normal, nonatrophic tubules of *furless* mice demonstrate that enzymatically active cathepsin L is required for quantitatively normal spermatogenesis.

MATERIALS AND METHODS

Animals

Mature male Sprague-Dawley rats and CD-1 mice were purchased from Charles River Laboratories (Indianapolis, IN). Mice heterozygous for the spontaneous mutation, *furless* (B6 × FSB/GnE a/a Fs/+), were purchased from Jackson Laboratories (Bar Harbor, ME) and homozygous *furless* mice and control littermates (wild-type or heterozygous) were obtained by breeding. Mature mice homozygous for the furless mutation were identified by the thinness of their fur and by their loss of all fur from patches of skin [10]. The G149R mutation in both alleles of the cathepsin L gene of furless mice [10] was confirmed by sequencing amplified genomic DNA. For the studies described in this paper, *furless* and control mice (wild-type or heterozygous for the *furless* mutation) were 110–120 days of age when testes were collected. *Furless* and control mice were obtained from the same litters. We did not attempt to distinguish the two genotypes of mice in the control group, wild-type mice heterozygous *furless*, because the two reported phenotypes of homozygous, *furless* and cathepsin L $^{-/-}$ mice, hair loss, and reduced numbers of CD4^{+} T cells, are not observed in heterozygous animals [13, 14]. The uses of animals for all experiments described herein were approved by the Johns Hopkins School of Public Health Institutional Animal Care and Use Committee.

Comparison of the Stage-Specific Expression of Cathepsin L mRNA by Mouse and Rat Sertoli Cells

Complementary DNA for mouse cathepsin L mRNA was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) using RNA from a CD-1 mouse kidney. Complementary DNA was synthesized from 5 μg of total RNA using random hexamers and the SuperScript Preamplification System (Gibco BRL, Rockville, MD). The cDNA for mouse cathepsin L mRNA was then amplified using 10% of the cDNA product and primers selected from the published sequence for mouse cathepsin L mRNA [15].

Forward primer:

5′-GACTGTATGGCACGAATGAGGAAG-3′
(nucleotides 218–241)

Reverse primer:

5′-AACTGGAGAGACGGATGGCTTG-3′
(nucleotides 878–855)

The PCR product was generated through 25 cycles for 3 min at 94°C, 1 min at 58°C, and 2 min at 72°C. This product was isolated by gel electrophoresis, ligated into the pCR II vector (Invitrogen, Carlsbad, CA), and the sequence of the PCR product was confirmed by DNA sequencing.

To synthesize antisense and sense mouse cathepsin L mRNAs, the plasmid was first linearized with *Hin*dIII or *Xba*I, respectively. ^{35}S-CTP and T7 or SP6 RNA polymerase was then used to synthesize antisense and sense RNA. Antisense mouse cathepsin L mRNA was used as a probe for Northern blot analysis of testis RNA and for in situ hybridization analysis of cathepsin L mRNA in testis sections [16]. Complete methods for the Northern blot analysis, for the processing of testes, for the synthesis of the riboprobes, for in situ hybridization, and for autoradiography have been previously published [16]. Sense and antisense rat cathepsin L mRNAs were synthesized as previously described [16].

Autoradiography was used to detect the binding of the riboprobes to testis sections. Following development of the autoradiograms, phase contrast and differential interference contrast microscopy were used to identify the tubules as being at stages VI, VII, VIII, or at other stages. Stages VI–VIII were identified based on the locations of elongate spermatids and residual bodies within the seminiferous epithelium. The autoradiograms were photographed using both brightfield and darkfield optics.

To quantify the hybridization of the antisense mouse or rat cathepsin L mRNAs to cross-sections of individual seminiferous tubules, areas of autoradiograms were photographed using brightfield optics, a Nikon Eclipse E800 microscope (Nikon, Inc., Melville, NY), a 20× lens and a Princeton charge-coupled device camera (Princeton Instruments, Trenton, NJ). IPLab Spectrum analysis software (Scanalytics, Fairfax, VA) was used to measure the optical density of silver grains over a minimum of ten 13-μm^2 areas along the base of each seminiferous tubule, where the specific autoradiographic signal was observed. The average optical density for each of these 10 areas was then calculated. Subsequently, the average background optical density obtained by hybridization of tissue sections with the sense strand probe was measured. Specific hybridization of the antisense probe to the tubules was defined by the corrected average optical density:

$$\text{corrected average optical density} = \text{average optical density}_{\text{antisense probe}} - \text{average background optical density}_{\text{sense strand probe}}.$$

The corrected average optical density was determined for nine seminiferous tubules at stage VII and nine seminiferous tubules at stages other than VI, VII, VIII (identified as "other" in Fig. 1).

Demonstration that Procathepsin L Is Secreted by Mouse Sertoli Cells

Sertoli cells were isolated from mature male mice and, as a control, from mature male rats and cultured as previously described [17]. Sertoli cells were cultured on a dried film of Matrigel (Becton-Dickinson Laboratories, Bedford, MA) in Ham F-12/Dulbecco modified Eagle medium (1:1) supplemented with human transferrin (5 μg/ml), insulin (10 μg/ml), and epidermal growth factor (1 ng/ml). After 3 days the cells were incubated for 16 h with ^{35}S-methionine (85 μCi/ml) in Ham F-12 with 0.45 μg/ml methionine. Radiolabeled proteins secreted by the Sertoli cells into the culture media were incubated with anti-rat procathepsin L or preimmune immunoglobulin (Ig) G. IgG-bound proteins were then resolved by SDS gel electrophoresis and detected by fluorography [5] The use of an antibody directed against rat procathepsin L to detect the mouse protein was appropriate because the amino acid sequences of the rat and mouse proteins are 94% identical and because the mouse and rat proteins are immunologically cross-reactive [6, 15, 18]. The anti-rat procathepsin L IgG used for immunoprecipitation was determined to be monospecific by the following criteria. The preparation of enzymatically active procathepsin L used as antigen produced a single immunoprecipitate by crossed immunoelectrophoresis [5]. A single radiolabeled protein, the size of procathepsin L, was immunoprecipitated from cell homogenates of Sertoli cells or stage VII seminiferous tubules, which had been incubated for 1 h with ^{35}S-methionine [5, 17]. When used for Western blot analysis of pituitary or testis extracts, this antibody detected both the proenzyme and the processed, mature enzyme form of cathepsin L ([8] and unpublished data).

Morphological Analysis of the Testes of Furless Mice

Control mice (110- to 120-day-old; wild-type or heterozygous for the *furless* mutation) and *furless* mice were anesthetized with pentobarbital, injected i.p. with 1000 U heparin and perfused sequentially via the left ventricle with Hepes-saline and 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). Tissues were postfixed with 1% osmium tetroxide, 1% potassium ferrocyanide, embedded in epon, cut into 1-μm sections, and stained with toluidine blue. Slides were examined using the Nikon Eclipse E800 microscope. Digital images were processed using PhotoShop software (Adobe Systems Inc., San Jose, CA)

The first question we asked was whether there was an increase in the numbers of tubules in *furless* mice that were undergoing atrophy. Tubules were defined as undergoing atrophy if they lacked one or more generations of spermatogenic cells. Seminiferous tubules of *furless* mice that were undergoing atrophy were morphologically characterized and the percentages of tubules undergoing atrophy in *furless* mice were compared with the percentage of tubules undergoing atrophy in age-matched controls (wild-type or heterozygous for the *furless* mutation). The second question we asked was whether spermatogenesis was quantitatively normal in tubules of *furless* mice that were not undergoing atrophy. To this end, we counted Sertoli cells (with visible nucleoli), preleptotene spermatocytes, type B spermatogonia, midpachytene spermatocytes, and round spermatids in all round to oval (length/width <1.5) cross-sections of stage VI, VII, and VIII seminiferous tubules of *furless* and control mice. Type A spermatogonia were also counted, but their numbers were too low for a meaningful statistical comparison. We also measured for each stage the nuclear diameters of each germ cell type and nucleolar diameters of Sertoli cells.

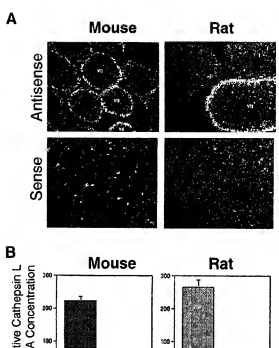

FIG. 1. Stage-specific expression of cathepsin L mRNA by mouse Sertoli cells. A) Darkfield microscopic examination of mouse and rat testis sections hybridized to antisense and sense, mouse and rat cathepsin L mRNAs, respectively. ^{35}S probes were used for this analysis and radioactivity was detected by autoradiography. Seminiferous tubules were identified as being at stages VI, VII, or VIII or other stages by a combination of phase contrast and differential interference microscopy. B) Quantitative analysis of cathepsin L mRNA concentration in seminiferous tubules at stage VII or at other stages (for the mouse, stages I–V and IX–XII; for the rat, stages I–V and IX–XIV) Relative concentrations of cathepsin L mRNA in mouse and rat tubules were estimated by measuring the optical density of silver grains over the base of individual seminiferous tubules. Data (mean ± SEM) for both mouse and rat tubules were obtained from nine separate seminiferous tubules at stage VII of the cycle and from nine separate tubules at the other stages noted above.

These diameters were used to calculate corrected numbers of each type of germ cell and Sertoli cell per tubule cross-section using the Abercrombie formula [19]:

$$\text{corrected cell counts} = \frac{\text{crude cell counts} \times \text{section thickness}}{\text{section thickness} + \text{nuclear diameter}}.$$

Because Sertoli cell nuclei have an irregular shape, nucleolar diameter and not nuclear diameter was used to calculate corrected Sertoli cell counts.

For each animal, cells were enumerated in a minimum of 15 tubules at stages VI, VII, or VIII and the data from these three stages were pooled. The average numbers of germ cells per Sertoli cell were calculated for each animal and used for statistical comparison of spermatogenesis in control and *furless* mice.

Statistical Analysis

ANOVA was used for all initial statistical comparisons. When more than two groups were compared and when ANOVA revealed overall significant differences between these groups, statistical differences between individual groups were tested using the Fisher least significant difference test. These analyses were performed using StatView software (SAS Institute Inc., Cary. NC).

RESULTS

Procathepsin L Is a Stage-Specific Secretory Product of Mouse Sertoli Cells

The objective of our first experiments was to determine whether mouse Sertoli cells shared two important characteristics with rat Sertoli cells, the stage-specific expression of the cathepsin L gene and the synthesis and secretion of procathepsin L. To determine whether the cathepsin L gene was transcribed in the mouse testis, 10 μg of RNA from mouse and rat testes were fractionated on denaturing agarose gel, blotted to nylon membranes, and hybridized with antisense mouse cathepsin L mRNA. This analysis detected predominant transcripts of 1.7 kilobases in both mouse and rat testes (data not shown). In situ hybridization was used next to test whether cathepsin L mRNA was expressed in vivo in the same stage-specific manner by mouse and rat Sertoli cells. Antisense mouse and rat cathepsin L mRNAs hybridized to the periphery of mouse and rat seminiferous tubules, respectively. Previous studies of rat testes demonstrate that Sertoli cell cytoplasm within the periphery of the tubules contains this transcript [6, 7, 16]. Lack of hybridization with the sense strand probe to seminiferous tubules demonstrated that hybridization of the antisense probe to RNA in the tubules was sequence specific (Fig. 1A). Detailed microscopic analysis of sections hybridized with the antisense probes indicated that in the testes of both rats and mice, maximal cathepsin L mRNA levels were observed at stages VI and VII of the cycle (Fig. 1A). In mice, cathepsin L mRNA levels were high in 85% of stage VII and in 50% of stage VI seminiferous tubules. Tubules at all other stages contained low levels of this transcript. In rats, high levels of cathepsin L mRNA were detected in all stage VI and VII seminiferous tubules but not in tubules at any other stage. Image analysis was next used to quantify the stage-specific changes in expression of this transcript by mouse and rat Sertoli cells. In mice, average cathepsin L mRNA levels at stage VII were approximately 5-fold higher than the average levels in tubules at stages I–V or IX–XII (Fig. 1B). In rats, expression of cathepsin L mRNA in stage VII tubules was approximately 10-fold higher than in tubules at stages I–V and IX–XIV.

To demonstrate that procathepsin L was secreted by mouse Sertoli cells, mature mouse Sertoli cells and control rat Sertoli cells were isolated, cultured for 3 days, and then incubated with ^{35}S-methionine. Radiolabeled proteins secreted by both mature rat and mouse Sertoli cells were incubated with monospecific anti-rat procathepsin L or preimmune IgG and the IgG-bound radiolabeled proteins fractionated by SDS gel electrophoresis. Fluorograms of the gels revealed that anticathepsin L IgG immunoprecipitated the same size secretory proteins from mouse and rat Sertoli cells (Fig. 2). In contrast, no proteins were immunoprecipitated by preimmune IgG.

The data from these experiments taken together demonstrate that mouse Sertoli cells express the cathepsin L gene in the same stage-specific manner as rat Sertoli cells. Furthermore, these results indicate that mouse Sertoli cells, like rat Sertoli cells, synthesize and secrete the proenzyme form of cathepsin L. Therefore, these findings validate the use of the *furless* mice to test the hypothesis that the stage-specific secretory product of Sertoli cells, procathepsin L, is required for quantitatively normal spermatogenesis.

Evidence of Increased Atrophy of Seminiferous Tubules of Furless Mice

As noted in the *Introduction,* the furless mouse has a point mutation in the cathepsin L gene that renders the enzyme catalytically inactive. To determine whether cathepsin L is required for quantitatively normal spermatogenesis, we examined the testes of 110- to 120-day-old mice. We chose to study animals of this age because cathepsin L is a major secretory product of mature but not immature Sertoli cells and because of the possibility that a testicular phenotype of *furless* mice might take some time to develop in mature animals [20]. Paired testis weights of 110- to 120-day-old *furless* mice (164 ± 12 mg; mean ± SEM) were significantly (25%) smaller than testes of age-matched controls (221 ± 7 mg). Microscopic examination revealed that all testes of *furless* mice had both normal seminiferous tubules (Fig. 3A) and morphologically abnormal seminiferous tubules that were undergoing atrophy (Fig. 3, B–G). This abnormal morphology was heterogeneous. Some tubules contained elongate spermatids but were missing both round spermatids and spermatocytes, (Fig. 3B), or only round spermatids (Fig. 3C) or pachytene spermatocytes (Fig. 3D). In contrast, no tubules were observed that were missing only preleptotene spermatocytes. Other morphologically abnormal tubules were missing elongate spermatids (Fig. 3E) or both elongate and round spermatids (Fig. 3F). Finally, a subset of tubules contained few if any germ cells (Sertoli cell-only; Fig. 3G). As a rule, normal tubules and tubules that were undergoing atrophy were found in the same area of the testis (Fig. 3H).

To quantify the extent to which lack of functional cathepsin L increased seminiferous tubule atrophy, we first enumerated in *furless* and control mice normal tubules and tubules that were undergoing all forms of atrophy (as defined in Fig. 3, B–G). This analysis (Fig. 4A) revealed that 18% of the tubules of *furless* mice were undergoing atrophy compared with 1.5% of tubules of control mice. We next asked whether one or more of the abnormal morphologies leading to seminiferous tubule atrophy were predominant. Such predominance might provide insight into events causing atrophy in animals that fail to express functional cathepsin L. Thus, we enumerated in each of the five *furless* mice apparently normal tubules and tubules exhibiting each of the abnormal morphology described above (Fig. 3, B–G). Results were then used to calculate the percentage of the tubules exhibiting each of the abnormal morphologies. This analysis (Fig. 4B) revealed no significant differences in the percentages of tubules exhibiting each type of abnormal morphology. These results taken together demonstrate that a 12-fold increase exists in atrophy of seminiferous tubules of 110- to 120-day-old *furless* mice and that no predominant abnormal morphology is exhibited by the tubules undergoing atrophy.

Seemingly Normal Tubules of Furless Mice Have Quantitatively Reduced Spermatogenesis

The increased incidence of seminiferous tubule atrophy in *furless* mice raised the question of whether lack of cathepsin L had a quantitative effect on spermatogenesis in seemingly normal, nonatrophic tubules. To address this question we determined the numbers per Sertoli cell of round spermatids, pachytene spermatocytes, preleptotene spermatocytes, and type B spermatogonia in stage VI–VIII tubules of control and *furless* mice. From these data we established the effect of cathepsin L on formation of preleptotene spermatocytes, the differentiation of these cells into pachytene spermatocytes, and the formation of round spermatids from pachytene spermatocytes. In performing this analysis we observed both type B spermatogonia and preleptotene spermatocytes in stage VII tubules. Therefore, in order to calculate the efficacy of the differentiation of preleptotene spermatocytes into pachytene spermatocytes, we estimated for each tubule cross-section the total numbers of preleptotene spermatocytes formed per Sertoli cell. This estimated number of cells was equal to the actual numbers of preleptotene spermatocytes per Sertoli cell plus twice the actual numbers of type B spermatogonia per Sertoli cell.

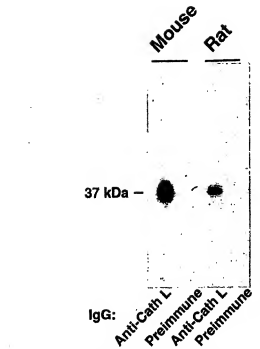

FIG. 2. Mouse Sertoli cells secrete procathepsin L. Sertoli cells were isolated from both mature CD-1 mice and from Sprague-Dawley rats, cultured for 3 days, and then incubated for 16 h with ^{35}S methionine. Proteins secreted into medium were then incubated with either anticathepsin L or preimmune IgGs, the IgG-bound proteins fractionated by SDS gel electrophoresis, and the radiolabeled proteins detected by fluorography. The size of procathepsin L obtained from both mouse and rat Sertoli cells was estimated by use of molecular weight standards.

Figure 5 shows the numbers of round spermatids and pachytene spermatocytes per Sertoli cell as well as the estimated total numbers of preleptotene spermatocytes formed per Sertoli cell in testes of *furless* mice and in age-matched controls. Note that our calculation of 13.6 round spermatids per Sertoli cell in stage VI–VIII tubules in control mouse testes is consistent with published results obtained with the optical dissector method, 12.5 step 1–XII spermatids per Sertoli cell [21]. Figure 5 demonstrates that lack of functional cathepsin L causes a significant reduction in numbers per Sertoli cell of round spermatids (68% of control) and pachytene spermatocytes (69% of control) and the estimated total numbers of preleptotene spermatocytes formed (84% of control). The actual number of preleptotene spermatocytes counted per Sertoli cells in stage VII and VIII tubules of *furless* mice was also significantly reduced (mean ± SEM: *furless,* 2.94 ± 0.32; control, 4.07 ± 0.2). These data also indicate that while all preleptotene spermatocytes of control mice gave rise to pachytene sper-

Normal
Missing Elongate Spermatids
Missing Round Spermatids and Spermatocytes
Missing Round and Elongate Spermatids
Missing Round Spermatids
Sertoli Cell-Only
Missing Pachytene Spermatocytes
Overview
A
B
C
D
E
F
G
H

matocytes, only 75% of the preleptotene spermatocytes of *furless* mice complete this process. In contrast, progression of pachytene spermatocytes to round spermatids was equally efficient (75%) in control and *furless* animals.

DISCUSSION

The studies described herein demonstrate that the proenzyme form of cathepsin L is a stage-specific secretory protein of mouse Sertoli cells, which is required either directly or indirectly for quantitatively normal spermatogenesis. Although both mouse and rat Sertoli cells in stage VII tubules express elevated levels of cathepsin L mRNA, the amplitude of the cycle of expression of this transcript by rat Sertoli cells is twice that of mouse Sertoli cells. In addition, while cathepsin L mRNA expression is elevated in all stage VI and VII tubules in rats, its expression is elevated in only 50% of stage VI tubules and 85% of stage VII tubules in mice. This later observation suggests that in mice, increased stage-specific expression of cathepsin L mRNA begins later in stage VI and ends sooner in stage VII than it does in rats. However, despite the quantitative differences in stage-specific expression of cathepsin L of mice and rats, these introductory experiments demonstrated that the mouse was an appropriate model to examine whether secretion by Sertoli cells of the stage-specific secretory product, procathepsin L, was required for quantitatively normal spermatogenesis.

We recognize that the studies presented herein do not unequivocally prove that the deficits in spermatogenesis of the *furless* mouse result directly from a lack of functional cathepsin L within the testis. It is possible that the testicular phenotypes of the *furless* mouse are due to the lack of functional cathepsin L in another organ that, in turn, has a deleterious effect on the testis. We consider this possibility unlikely, however. As argued at the end of this discussion, the morphology of the testes of *furless* mice does not suggest insufficient support of spermatogenesis by testosterone. In addition, data presented in this and published papers indicate that the phenotypic effects of the *furless* mutation are restricted to the testis, skin, and thymus [10]. Thus, we interpret the testicular morphology of *furless* mice as resulting from the secretion by Sertoli cells of a procathepsin L that is not a precursor to a functional protease. This interpretation predicts that procathepsin L has a biologically important function within the seminiferous epithelium. In *furless* mice, the lack of functional cathepsin L has two distinct consequences, atrophy of a subset of seminiferous tubules and quantitatively reduced spermatogenesis in seemingly normal, nonatrophic tubules.

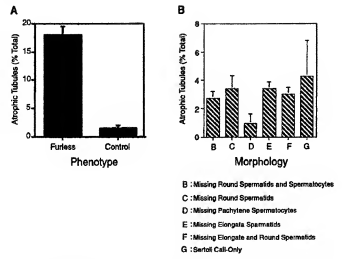

FIG. 4. Characterization of atrophy of seminiferous tubules in *furless* mice. A) Comparison of percentages of tubules in *furless* and control mice undergoing atrophy. Data (means ± SEM) are expressed as a percentage of all tubules that are undergoing atrophy. B) Breakdown of the types of seminiferous tubule atrophy in *furless* mice. Data (means ± SEM) are expressed as a percentage of all tubules of *furless* mice that exhibit a specific abnormal morphology. The letters (B–G) refer to the different abnormal morphologies depicted in Figure 3.

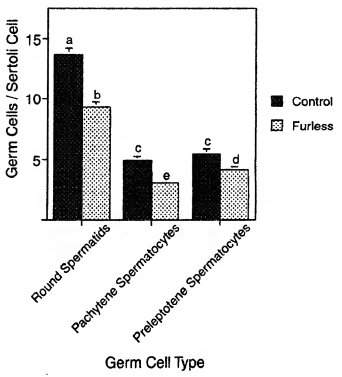

FIG. 5. Numbers of round spermatids, pachytene spermatocytes, and estimated total numbers of preleptotene spermatocytes per Sertoli cell in nonatrophic tubules of control and *furless* mice. Data (means ± SEM) labeled with different letters differ statistically.

FIG. 3. The morphology of seminiferous tubules in testes of 110- to 120-day-old *furless* mice. *Furless* mice contained both normal seminiferous tubules and seminiferous tubules that were undergoing atrophy. The morphologies of the tubules that were undergoing atrophy were heterogeneous. A) A normal seminiferous tubule in the testis of a *furless* mouse. B) A seminiferous tubule missing round spermatids and spermatocytes but containing elongate spermatids (marked by a white arrow). C) A seminiferous tubule missing round spermatids but containing elongate spermatids and pachytene spermatocytes. The black arrow points to a residual body. D) A seminiferous tubule missing pachytene spermatocytes but containing elongate and round spermatids. E) A seminiferous tubule missing elongate spermatids. The white arrow points to one of four apoptotic pachytene spermatocytes. F) A seminiferous tubule missing both round and elongate spermatids. G) A seminiferous tubule with Sertoli cell-only morphology. The black arrow points to the nucleus of a Sertoli cell. H) An overview of a testis of a *furless* mouse that shows the presence of normal tubules and atrophic tubules (marked by triangles) in the same region of the testis. The black horizontal bars in the lower left-hand corner of each figure are equal to 10 μm in the original testis sections.

Evidence That Secretion of Procathepsin L by Normal Sertoli Cells Prevents Atrophy of the Seminiferous Epithelium

Our results demonstrate that 110- to 120-day-old *furless* mice exhibit a 12-fold increase in the numbers of tubules undergoing atrophy. Because substantial atrophy was not observed in 60-day-old *furless* mice (unpublished results) the lack of functional cathepsin L may have a cumulative, deleterious effect on this epithelium. The heterogeneous morphologies of regressing tubules of 110- to 120-day-old *furless* mice suggest that multiple pathways lead to the Sertoli cell-only phenotype. Tubules that contained elongate spermatids but lacked earlier germ cells (see Fig. 3, B–D) may have experienced apoptotic death of spermatocytes or round spermatids. In contrast, sloughing or phagocytosis of elongate spermatids may contribute to the atrophy of other tubules (see Fig. 3, E and F). What is striking however, is the absence of round spermatids in more than 50% of the tubules that were undergoing atrophy. In contrast, we never observed a tubule that lacked only preleptotene spermatocytes. Thus, once atrophy of the seminiferous tubules of *furless* mice commences, the lack of functional cathepsin L has a marked deleterious effect on formation or survival of haploid germ cells. In contrast, in the seemingly normal tubules of *furless* mice, lack of cathepsin L has deleterious effects on earlier phases of spermatogenesis. This argues that in wild-type animals, cathepsin L acts directly or indirectly through different mechanisms to suppress atrophy of seminiferous tubules and to support quantitatively normal spermatogenesis.

The question remains, why do only 18% of the tubules of *furless* mice undergo atrophy? It should be noted that similar findings have been reported in other studies of testicular atrophy. In 18-mo-old Brown Norway rats, approximately 50% of the seminiferous tubules lack germ cells, whereas most of the other tubules are morphologically normal [22]. Mild restriction of testicular blood flow or 1 h of testicular torsion induces germ cell apoptosis in only a subset of seminiferous tubules [23, 24]. Heating of the testis causes germ cell apoptosis in a stage-specific manner [25]. Thus, there appears to be a differential response of seminiferous tubules to physiological insults that cause germ cell death. Such differential sensitivity may explain why only some tubules are atrophic in mature, *furless* mice.

Another potential reason for atrophy of only a subset of tubules is that Sertoli cells express at least two other members of the cathepsin family of proteases, cathepsin A and cathepsin D [26, 27]. These two proteases may partially compensate for the lack of functional cathepsin L and temporally spread out the deleterious effects in *furless* mice that lead to atrophy of the seminiferous tubules.

Lack of Functional Cathepsin L Results in Reduced Formation of Preleptotene Spermatocytes and Their Differentiation into Pachytene Spermatocytes

Enumeration of spermatogenic cells revealed that cathepsin L is required for quantitatively normal spermatogenesis. The average, nonatrophic tubules of *furless* mice contained 32% fewer round spermatids per Sertoli cell than the average tubule of control mice. This is a significant reduction in numbers of round spermatids per Sertoli cell when compared to spermatogenesis in mice that lack functional genes for the FSHβ subunit (42% reduction) or the activin type II receptor (28% reduction) [21]. The reduction in formation of round spermatids in *furless* mice resulted from lesions at two different phases of spermatogenesis. Our estimation of the total numbers of preleptotene spermatocytes formed per Sertoli cells indicates that the lack of functional cathepsin L results in a 16% reduction in numbers of these cells. Thus, cathepsin L must directly or indirectly affect numbers or differentiation of stem spermatogonia or suppress apoptosis of differentiated spermatogonia. The second lesion in the *furless* mouse occurred during meiosis. Figure 5 indicates that in control mice, few if any spermatocytes were lost as they progressed from the preleptotene to the pachytene stages of meiosis. In contrast, 25% of spermatocytes in *furless* mice were lost during this process. This loss of cells is consistent with the presence of apoptotic midpachytene spermatocytes in stage VII tubules of *furless* mice (see Fig. 3E). Cathepsin L may not affect later phases of spermatogenesis, however. In contrast to the differences in ratios of less-mature spermatogenic cells, ratios of round spermatids to pachytene spermatocytes were identical in *furless* and control animals. Thus, cathepsin L affects the formation and differentiation of spermatocytes but not the completion of meiosis or progression of spermatids to step 7 of spermiogenesis. Although step 16 spermatids were not enumerated in the present study, the presence of these cells in stage VII tubules of *furless* mice provides proof that spermiogenesis was completed in these animals. Thus, cathepsin L affects a different phase of spermatogenesis than does testosterone, which primarily regulates the completion of spermiogenesis and spermiation [28, 29]. This argument supports the concept that the testicular phenotype in *furless* mice is not due to a physiologically significant deficit in intratesticular testosterone levels.

In summary, this study provides evidence that the stage-specific secretion of procathepsin L by Sertoli cells has two different consequences within the seminiferous epithelium. Secretion of this stage-specific product prevents atrophy of seminiferous tubules. Our analysis of atrophic tubules of *furless* mice suggests that cathepsin L prevents atrophy in part by preventing the loss of round spermatids. Cathepsin L also has a role in the normal seminiferous epithelium where it directly or indirectly promotes the formation of preleptotene spermatocytes and their differentiation into pachytene spermatocytes.

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